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Preventing Prostate Cancer Metastasis by Targeting Exosome Secretion

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Table of Contents

1. Introduction	4
2. Keywords	4
3. Overall Project Summary	4
Current Objectives	4
Results and Progress	4
Accomplishments and Discussion.....	6
Anticipated changes	7
4. Key Research Accomplishments	7
5. Conclusion	7
6. Publications, Abstracts, and Presentations	7
7. Inventions, Patents and Licenses	7
8. Reportable Outcomes.....	7
9. Other Achievements	7
10. References	7
11. Appendices	7

1. Introduction

Proposal abstract. We hypothesize that exosomes secreted by advanced prostate cancer cells groom stromal cells at pre-metastatic sites to render the bone microenvironment more favorable for metastatic growth. We generated this hypothesis by using a probabilistic gene functional network to re-analyze a publicly available dataset in which transcript levels of androgen-responsive genes are compared between early- and advanced/metastatic prostate cancer [1]. The hypothesis is also supported by recent findings in exosomes secreted by melanoma cells [2]. We propose to test the hypothesis that exosomes derived from advanced stage prostate cancer exert a pro-metastatic influence on bone stromal cells. **Aim #1:** We will perform a quantitative comparison of changes in the structure and cargo of exosomes derived from early versus advanced prostate cancer cell lines to identify mechanisms of action (using large-scale, high-resolution proteomics). **Aim #2:** We will profile alterations in potential target stromal cells upon exposure to early versus late prostate cancer exosomes to determine the effect on the metastatic microenvironment (using next-generation sequencing and proteomics).

2. Keywords

Exosomes; proteomics; ultracentrifugation; secretome; prostate cancer; metastasis

3. Overall Project Summary

This report is an annual report for a DOD Hypothesis Testing Award (1 year, \$75,000 direct costs).

Current Objectives

To test the above-stated hypothesis in a robust and quantitative experimental setup, we set the following objectives:

1. Establish cell line growth in conditions amenable to exosome studies
2. Establish exosome isolation protocol to quantitatively extract exosomes for (i) analysis by proteomics and (ii) treatment of bone marrow cells
3. Characterize extracted exosomes with respect to exosome markers and the proteomic profile
4. Conduct pilot studies for treatment of bone marrow cells with exosomes extracted from media conditioned by prostate cancer cells

Results and Progress

As specified in the approved no-cost extension, the project was delayed due to loss of key personnel and medical leave of the PI. Despite these challenges, we made progress in several ways.

1. We established growth of two prostate cancer cell lines LnCAP (non-metastatic) and LNCaP C4-2b (metastatic). To avoid contamination by exosomes from the fetal bovine serum (FBS) supplement that is part of mammalian cell growth media, we tested if the cells grow in FBS-free medium. As the cells did not grow well (*not shown*), we tested an alternative method in which cells were grown in medium with exosome-depleted FBS. Cells grew well with normal phenotype (*not shown*). We also established growth of two bone marrow cell lines (HS-27A and

hFOB1.19) in normal medium supplemented with FBS-free medium. Again, the cells showed normal phenotypes (*not shown*).

2. The extraction of exosomes from growth medium is not well-established. Most protocols and kits are optimized for use of either serum and urine. In addition, for the purpose of our experiments, we required protein samples from isolated exosomes to be compatible with mass spectrometry analysis which posed additional challenges.

We tested four different commercially available exosome isolation kits (**Table 1**). The kits use a variety of different approaches and work with 4 to 10ml of cell culture medium.

To test the ability to extract exosomes in a quantitative manner and preserve exosomal proteins, we used both protein gels (**Figure 1**) and mass spectrometry to analyze the samples (*not shown*). **Figure 1** shows that the Invitrogen, ExoQuick, and ExoSpin kits provide protein samples covering a wide range of molecular weights. The three kits are also quantitative enough to provide enough protein sample for mass spectrometry analysis. However, due to the kit-specific isolation protocols, the samples still need to be processed further before they are safe to use for mass spectrometry.

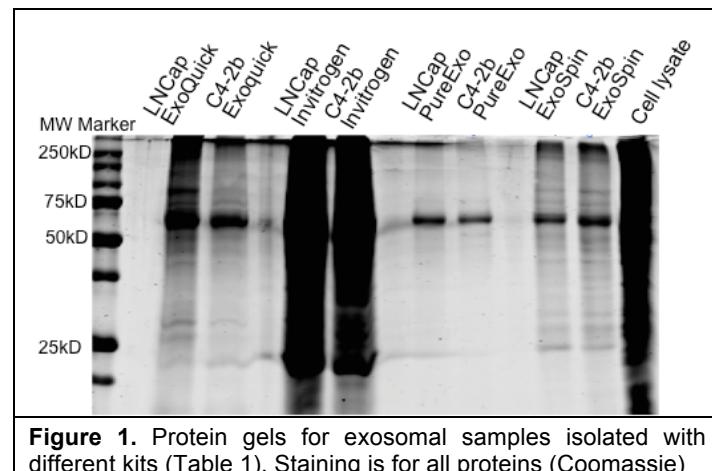


Figure 1. Protein gels for exosomal samples isolated with different kits (Table 1). Staining is for all proteins (Coomassie)

Table 1. Exosome isolation kits tested

Name of the kit used	Amount of Starting Material	Preparation/ Methods	Preparation Time	End Sample Amount
ExoQuick by System Biosciences	10mL of Cell Culture Media + 2 mL of ExoQuick-TC Exosome Precipitation Solution	Centrifugation (removal of cell debris) Overnight incubation (4 degrees) Centrifugation	- Day 1: 20- 30 minutes - Day 2: 45-60 minutes	100-500uL
Total Exosome Isolation Reagent by Invitrogen	10mL of Cell Culture Media + 5mL of Total Exosome Isolation Reagent	Centrifugation (removal of cell debris) Overnight incubation (4 degrees with rotation) Ultracentrifugation	- Day 1: 45-60 minutes - Day 2: 75-90 minutes	100uL
Pure Exo by 101 Bio	4mL of Cell Culture Media+ 1mL (Solutions A+B+C)	Centrifugation (removal of cell debris) Vortexing 4 degree incubation Column separation/purification	- 1-2 hours	50-200uL
ExoSpin by Cell Guidance Systems	6mL of Cell Culture Media + Buffer A (2mL)	Centrifugation (removal of cell debris) Overnight incubation (4 degrees with rotation) Ultracentrifugation Column purification	- Day 1: 45-60 minutes - Day 2: 2 hours	200uL

3. To validate the specificity of exosome isolation of the four kits, we used established exosome markers (CD9, CD81, Hsp70) in western blot experiments (**Figure 2**). The ExoQuick and ExoSpin kits are positive for all three markers without extensive unspecific bands; the ExoSpin kit provides the most consistent results combined with quantitative protein extraction over a large range of molecular weights (see item 2, **Figure 1**). Therefore, we will use this kit for subsequent protocol optimization.

We also conducted preliminary mass spectrometry studies with the isolated exosomes. However, we identified only few proteins due to sample contamination with polymers that originated from the isolation kits. We are working with the companies to address these issues, i.e. remove the polymers prior to mass spectrometry analysis. Encouragingly, our preliminary proteomics analysis of prostate exosome samples identified several typical exosome markers, as defined by Exocarta (http://www.exocarta.org/exosome_markers).

These proteins are listed in **Table 2**.

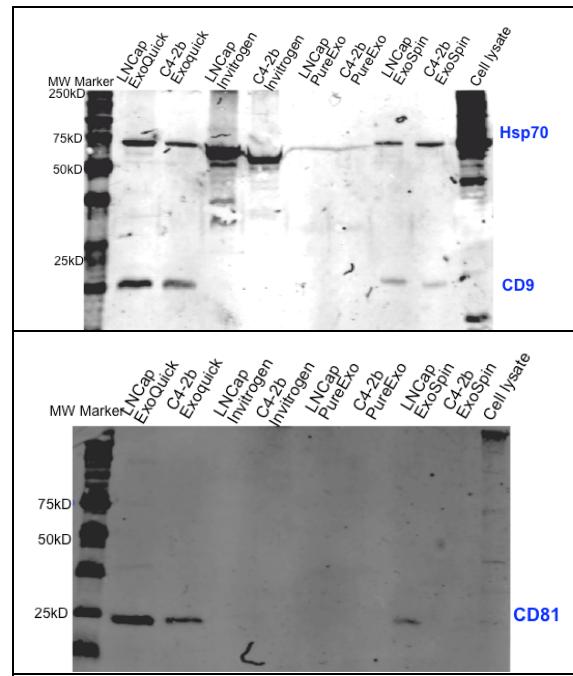


Figure 2. Western blots with antibodies against four exosome markers

Table 2. Exosome markers identified by mass spectrometry

Gene	Peptide counts (all)	Protein names
CD81	1;1;1;1;1;1;1;1;1	CD81 antigen
HSPA5	1;1;1;1;1;1;1;1;1;1;1;1;1;1;1;1;1;1	78 kDa glucose-regulated protein;Heat shock cognate 71 kDa protein;Heat shock 70 kDa protein 1-like;Heat shock-related 70 kDa protein 2
GAPDH	1;1;1;1	Glyceraldehyde-3-phosphate dehydrogenase
SDCBP	2;2;1;1;1	Syntenin-1
HSP90AA1	2;2;2;1;1;1;1;1;1;1;1;1;1;1	Heat shock protein HSP 90-alpha;Heat shock protein HSP 90-beta;Putative heat shock protein HSP 90-beta 2;Putative heat shock protein HSP 90-alpha A2

4. Due to the difficulties with the commercially available exosome isolation kits, we explored alternative ways to test the proposal's hypothesis. One route includes a pilot study in which we use the *secretome*, i.e. the entirety of the secreted protein samples, including exosomes and other extracellular vesicles, to treat bone marrow cells. We will then test the cells for commonly used markers of bone metastasis or a pre-metastatic state, i.e. MET from ref. [2], α -6 and α -2 integrin, ALP and endothelin-1. We are in the process of conducting this experiment (*results not shown*).

Accomplishments and Discussion

We have made substantial progress towards establishing the protocols needed for robust and quantitative analysis of the effect of exosomes derived from metastatic prostate cancer cells on bone marrow cells. Due to complications with existing, commercially available isolation kits, we have started to explore alternative approaches. We will use these protocols to test the molecular composition of these exosomes and their effect on bone marrow cells with respect to their conditioning towards a pre-metastatic environment.

Anticipated changes

While we will continue our efforts to isolate pure exosomes, we will investigate routes to analyze the entire secretome (which includes exosomes). Such an analysis would have the advantage that other non-exosomal proteins and vesicles that may function to condition bone marrow cells to a pre-metastatic state will be included – therefore expanding the scope of the original hypothesis. Protocols to establish quantitative isolation and analysis of the secretome are available in literature [3, 4] and we have already communicated with the main author of these papers (J. Krijgsveld, EMBL Germany).

4. Key Research Accomplishments

Nothing to report.

5. Conclusion

The proposed work tests the effect of exosomes derived from metastatic prostate cancer cells on bone marrow cells. This work has direct relevance towards an understanding of the often lethal complications of prostate cancer, manifested in extensive and painful metastasis of the bone. The work will lay the foundation for development of drugs to target advanced prostate cancer.

6. Publications, Abstracts, and Presentations

Nothing to report.

7. Inventions, Patents and Licenses

Nothing to report.

8. Reportable Outcomes

Nothing to report.

9. Other Achievements

Nothing to report.

10. References

1. Hendriksen, P.J., et al., *Evolution of the androgen receptor pathway during progression of prostate cancer*. Cancer Res, 2006. **66**(10): p. 5012-20.
2. Peinado, H., et al., *Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET*. Nat Med, 2012. **18**(6): p. 883-91.
3. Eichelbaum, K. and J. Krijgsveld, *Combining pulsed SILAC labeling and click-chemistry for quantitative secretome analysis*. Methods Mol Biol, 2014. **1174**: p. 101-14.
4. Eichelbaum, K., et al., *Selective enrichment of newly synthesized proteins for quantitative secretome analysis*. Nat Biotechnol, 2012. **30**(10): p. 984-90.

11. Appendices

N/A